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TITLE: Early Diagnosis of Clear Cell Kidney Cancer via VHL/HIF Pathway-regulated Circulating microRNA

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The over-arching objective of this research project is to identify a panel of diagnostic miRNAs that are measurable in the serum and will be able to identify kidney cancer in its earliest stages. We hypothesized that serum levels of miR-210 would be significantly elevated in patients with clear cell renal carcinoma and that a panel of serum miRNAs could be defined and optimized for use as a diagnostic tool in patients presenting with a renal mass. To achieve this objective, we proposed three specific aims to test our hypotheses, Institutional IRB approvals have been obtained (specific aim 1 task 1). Retrospectively collected samples have been transferred and analyzed (specific aim 1 task 3). Milestones are on track and we do not anticipate any alterations to our SOW

15. SUBJECT TERMS- none provided

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DOD Idea Award: Collaborative Option – Year 2 Progress Report

Early Diagnosis of Clear Cell Kidney Cancer via VHL/HIF Pathway-Regulated Circulating microRNA

Award Number W81XWH-11-1-0714

Initiating PI: Muneesh Tewari, MD, PhD (Fred Hutchinson Cancer Research Center)
Partnering PI: Allan Pantuck, MD (University of California, Los Angeles)

INTRODUCTION:

The over-arching objective of this research project is to identify a panel of diagnostic miRNAs that are measurable in serum and will be able to identify kidney cancer in its earliest stages. We hypothesized that serum levels of miR-210 would be significantly elevated in patients with clear cell renal carcinoma and that a panel of serum miRNAs could be defined and optimized for use as a diagnostic tool in patients presenting with a renal mass. To achieve this objective we proposed three Specific Aims to test our hypotheses. As previously reported, in Year 1 we focused on Aim 1 in which we evaluated the utility of miR-210 as a marker for clear cell renal carcinoma. In an initial sample set of retrospective samples we found significant differences in the level of miR-210 in clear cell renal cases as compared to control serum samples. We also made significant progress in establishing digital PCR for miRNA detection in this sample set. During the current reporting period (Year 2) we utilized our sample set in global miRNA profiling studies as well as in confirmation studies of our previous miR-210 data using digital PCR. We have identified an eight miRNA marker panel for analysis in prospectively collected samples in the upcoming 3rd year of this project.

BODY:

Specific Aim 1: Evaluate the HIF-induced miRNA, miR-210, as a circulating marker for clear cell renal carcinoma detection in a retrospective cohort of patients presenting with a renal mass.

Tasks 1-6: Completed in Year 1

Task 7: Digital PCR analysis of miR-210 and control miRNAs in retrospectively collected specimens. (Tewari/FHCRC; months 15-18)
AND
Task 8: Data analysis to determine performance of miR-210 in retrospective samples and to compare results of qRT-PCR vs. Digital PCR for measurement of circulating miRNA. (Tewari and Mcintosh/FHCRC; months 18-21)

As described in our previous report we extracted RNA from a retrospectively collected specimen set consisting of serum samples from 20 non-RCC controls and 42 RCC cases. Using qRT-PCR we determined the level of miR-210 in these samples. As previously reported, 14 of our case samples proved to be unusable. Consequently, we proceeded to analyze miR-210 levels in the 20 controls and 28 cases. We reported a significant difference in the levels of miR-210 between cases and controls, with case samples having higher levels of miR-210 (**Figure 1**, left

panel). During the current reporting period we utilized these same specimens to examine the level of miR-210 using digital PCR. **Figure 1** presents the digital PCR data (right panel) as compared to the previously reported qRT-PCR data (left panel). As shown, we confirmed by digital PCR a significant difference in the level of miR-210 in case samples relative to controls, with cases having on average about 2.5-fold greater serum miR-210 than controls. Receiver Operating Curve (ROC) analysis demonstrated the ability of serum miR-210 to distinguish cases from controls and the AUC was in fact slightly better with digital PCR than with standard qRT-PCR. We have further subdivided the data so that clear cell histology cases are analyzed separately from non-clear cell cases, and compared separately to controls. qRT-PCR data is presented on the left while digital PCR data is presented on the right in **Figure 2**. We find the greatest difference in miR-210 levels to be between clear cell and control samples, although miR-210 levels are also higher overall even in non-clear cell kidney cancer patients compared with controls, suggesting that this marker could be useful for more comprehensive detection of kidney cancer than had been originally hypothesized. This conclusion at this point is tempered by the limited number of non-clear cell samples studied (i.e., n=5).

Specific Aim 2: Discover circulating miRNA markers that are complementary to miR-210 for clear cell renal carcinoma detection, for assembly into a biomarker panel.

Task 1: Global profiling of miRNAs in a subset of retrospective blood specimens, described in Aim 1, using microfluidic Taqman miRNA qRT-PCR array platform. (Tewari/FHCRC; months 12-18)
AND
Task 2: Data analysis to identify the best performing miRNA biomarkers from Task 1, assessed both individually and in combination with miR-210. (Tewari - McIntosh/FHCRC; months 19-21)

For this Task we used miRNA profiling qRT-PCR arrays (Exiqon, Inc.) to globally profile miRNA expression in our specimen sets with the aim of identifying additional circulating miRNAs that may serve as diagnostic markers for clear cell renal carcinoma. Using the same samples as in Aim 1, a universal reverse transcription reaction for use with the Exiqon miRNA array panels was performed for each RNA sample. Expression of 378 human miRNAs was profiled on these arrays by qRT-PCR using locked nucleic acid-based primers to achieve high technical sensitivity and specificity. Of these, 19 miRNAs were undetected in all samples. Quality control of the array data was achieved using a number of spike-in and inter-plate calibrators provided with the arrays. As demonstrated in **Figure 3,** with the exception of a few UniSP6 outliers, the controls performed well and we did not observe any significant batch or edge effects which could negatively affect the quality of the data.

We then performed differential miRNA expression analysis and the results of top differentially abundant serum miRNAs between cases and controls are shown in **Figure 4.** This data has been normalized using the median UniSp6 value and undetermined samples have been set to a Ct of 40. Depicted in **Figure 4A** are the top 5 miRNAs overexpressed in kidney cancer cases relative to control samples, chosen on the basis of greatest fold difference in expression (i.e., greatest difference in Ct values of cases vs. control). These miRNAs, all of which show a greater than 4-Ct elevation (i.e., greater than 16-fold increased abundance) in cases comprise miR-133a, -193b, -452, -1 and -365. In **Figure 4B** the top 5 overexpressed miRNAs as determined by t-test p-value are displayed. These include three of the miRNAs with the greatest fold difference (miR-133a, -452 and -1) from **Figure 4A**, as well as two additional miRNAs (miR-143 and -218). We also analyzed the profiling data with respect to miR-210 **(Figure 5).** While our results do suggest a difference between cases and controls, the differences are not as

striking as seen using the same samples in individual Taqman qPCR and digital PCR assays (Aim 1). This finding is not completely unexpected, as we frequently find individual Taqman assays utilizing assay specific reverse transcription primers to show superior performance than assays performed in a bulk reaction using a universal primer, as is required for miRNA profiling. The analysis of prospectively collected samples in Aim 3 will ultimately provide the best validation of serum miR-210 and the seven additional markers identified to date.

Task 3: Confirmation of up to 10 top-ranking miRNAs identified in Task 2. (Tewari/FHCRC; months 22-26)

At this point we are planning to use the validation set in Aim 3 for both confirmation and validation, as it will provide the most rigorous confirmation of the candidate biomarker panel that is emerging from Aim 2.

Specific Aim 3: Prospectively validate a circulating miRNA panel for the pre-operative diagnosis of clear cell renal carcinoma in patients with renal mass.

Task 1: Unselected, prospective blood collection from 150 patients presenting with renal mass (IRB approval and Material Transfer Agreement to be obtained under Aim 1, Task 1 above). (Pantuck/UCLA; months 7-29)

Prospective collection of serum samples is ongoing at UCLA by Dr. Pantuck at UCLA and as indicated in our SOW we anticipate sample collection to continue into Year 3 of this project.

Task 2: Transfer of unselected prospectively collected blood specimens for miRNA panel cross-validation. (Pantuck/UCLA; months 7-29)

We plan to transfer prospectively collected samples from UCLA to Fred Hutchinson Cancer Research Center once the specimen collection is completed, in order to avoid confounding batch effects that we have found can occur if specimens are not analyzed together. We expect to receive the specimens during Year 3 of this award and perform cross-validation of our miRNA marker panel as planned.

KEY RESEARCH ACCOMPLISHMENTS:

- We have confirmed and validated our previous qRT-PCR data via digital PCR, establishing that serum samples from patients with clear cell renal carcinoma contain elevated levels of miR-210.
- We have profiled the global expression level of 378 human miRNAs in our sample set and identified 7 miRNAs, in addition to miR-210, that are substantially elevated in our case samples relative to control samples.
- We are continuing our prospective blood collection (by Dr. Pantuck at UCLA) for biomarker panel validation.

REPORTABLE OUTCOMES:

- Manuscripts/Abstracts/Presentations: We recently published the following manuscript: Hindson CM, Chevillet JR, Briggs H, Gallichotte E, Ruf IK, Hindson BJ, Vessella RL, Tewari M. Absolute quantification of low abundance targets by droplet digital PCR versus analog real-time PCR. *Nature Methods*, doi: 10.1038/nmeth.2633, e-published ahead of print, 2013.
- Patents and licenses applied for and/or issued: None in the current reporting period
- Development of cell lines, tissue or serum repositories: None in the current reporting period
- Informatics such as databases and animal models, etc.: None in the current reporting period
- Funding applied for based on work supported by this award: None in the current reporting period
- Employment or research opportunities applied for and/or received based on experience/training supported by this award: None in the current reporting period

CONCLUSION:

In summary, our efforts to date have been focused on evaluating the utility of circulating miR-210 as a diagnostic marker for clear cell renal carcinoma and on identifying additional differentially expressed miRNAs which may be assembled into a diagnostic screening panel. These efforts were initiated on serum from a retrospective cohort of patients that included 42 RCC patients, of which 23 were of the clear cell histology, and 20 control individuals. Analysis of miR-210 levels in this sample set revealed a significantly higher level of miR-210 in serum from the RCC sample set as compared to the control set. These results, generated using standard qRT-PCR, were further confirmed using digital PCR. The most significant difference in miR-210 levels was observed in the comparison of clear cell cases to controls, although non-clear cell case also had higher levels of miR-210 than controls, suggesting that this marker may have value in distinguishing kidney cancer cases more broadly from controls. Global miRNA expression profiling identified several additional candidate serum miRNA markers which are present at higher abundance in RCC cases, which together with miR-210 represent a panel of markers that we plan to evaluate in Year 3 of this project using a prospective blood specimen set being collected by Dr. Pantuck at UCLA. We remain on-track for reaching our overall goal of discovering and validating a circulating miRNA panel for blood-based RCC diagnosis.

REFERENCES: None cited.
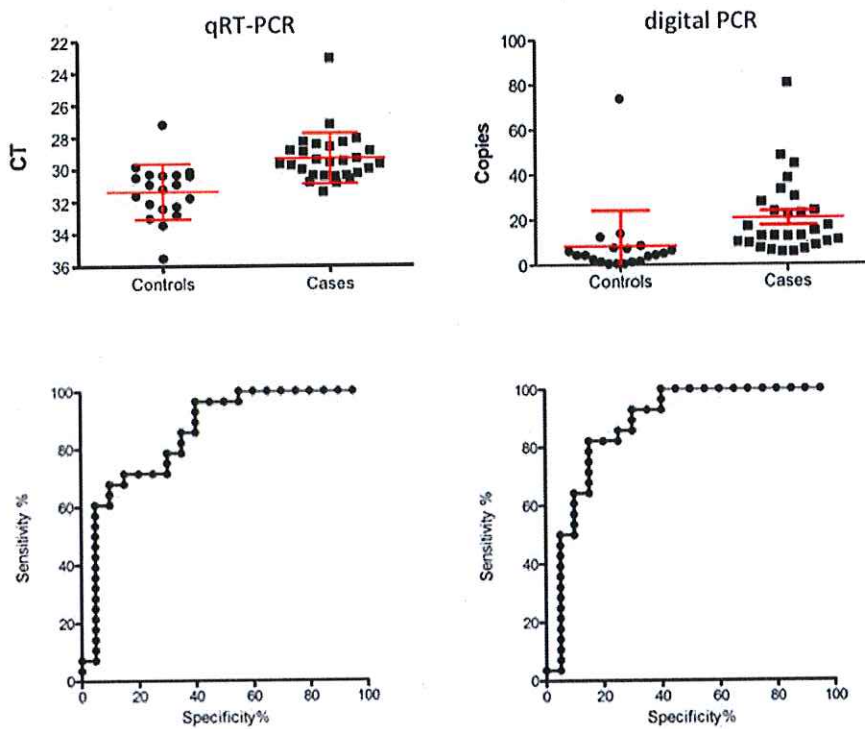
SUPPORTING DATA:

Figure 1: miR-210 levels in 28 RCC cases vs. 20 control serum samples with normalization using spiked-in cel-miR-39 RNA oligonucleotide. **Left, top panel:** miR-210 levels as measured by standard qRT-PCR. Y axis has been inverted to indicate that high CT values=low copy number and low CT values=high copy number. **Right, top panel:** miR-210 copy number as measured using digital PCR. **Bottom panels:** show the corresponding Receiver Operating Characteristic (ROC) curves. Area Under the Curve (AUC) for qRT-PCR is 0.85 and for digital PCR is 0.88.
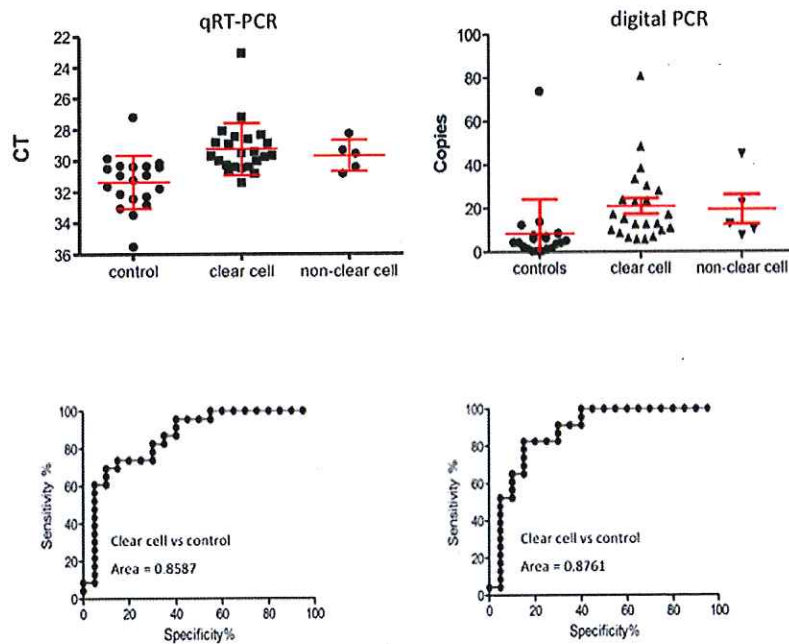
Figure 2: Further analysis of data presented in Fig.1 subdivided into clear cell and non-clear cell renal carcinoma and control samples. As in Fig. 1, left panel depicts qRT-PCR data while right panel depicts droplet digital PCR data, and bottom panels show ROC curves. "Area" refers to AUC of the ROC curve.
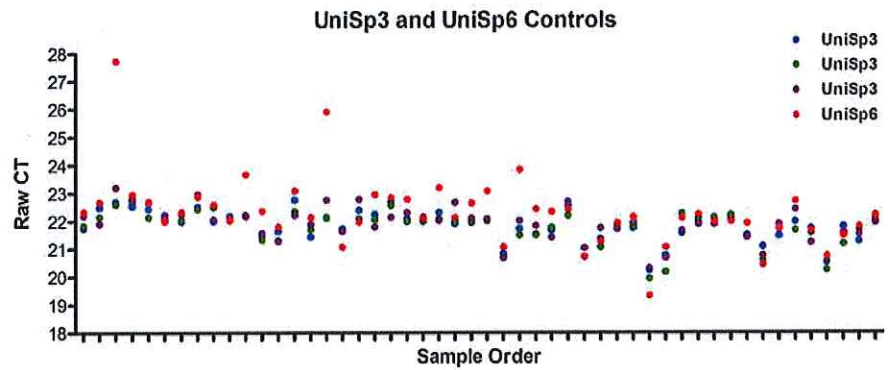
Figure 3: Global miRNA expression profiling was performed on our case-control sample set. Depicted here are the values obtained for control RNAs used to normalize miRNA expression data from sample-to-sample and plate-to-plate. Sample order is indicative of the order in which samples were processed (randomly mixed cases and controls).
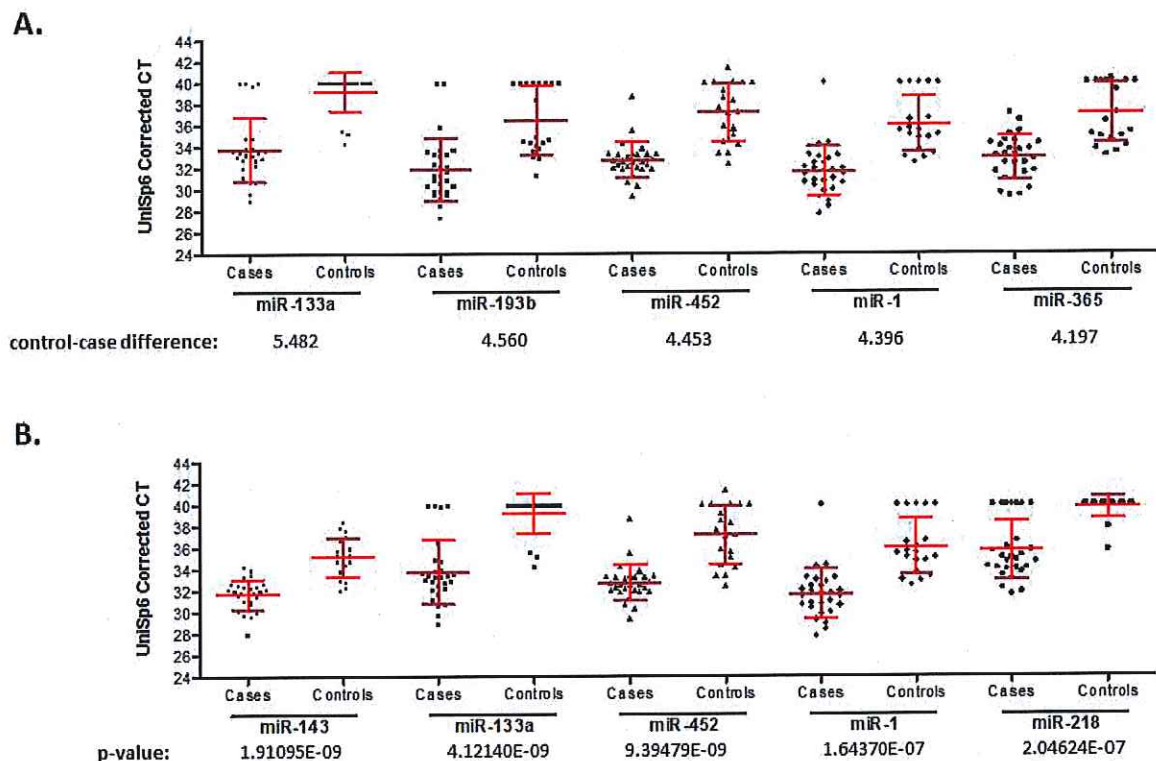
A.

control-case difference:

	miR-133a	miR-193b	miR-452	miR-1	miR-365
	5.482	4.560	4.453	4.396	4.197

B.

	miR-143	miR-133a	miR-452	miR-1	miR-218
p-value:	1.91095E-09	4.12140E-09	9.39479E-09	1.64370E-07	2.04624E-07

Figure 4: Global miRNA expression profiling reveals a number of miRNAs overexpressed in RCC cases relative to control serum samples. **(A)** Data presented for the top 5 miRNAs which show the greatest absolute differences in abundance between average case and average control CT values. **(B)** Data presented for the top 5 miRNAs overexpressed in cases as defined by lowest t-test p-value (unadjusted).
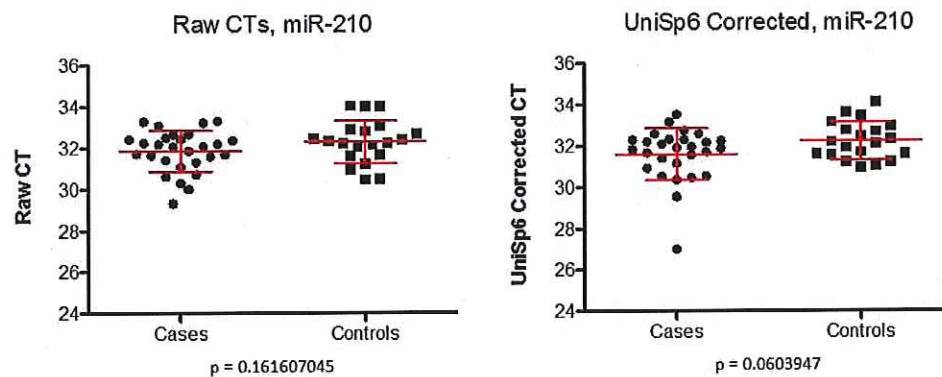
Figure 5: miR-210 levels in RCC case and control samples as determined by global miRNA profiling. **Left panel:** Raw CT values obtained for miR-210. **Right panel:** CTs for miR-210 following correction of data via normalization using values obtained for UniSp6.